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Critical Gestational Day of Teratogenesis by Di-*n*-butyltin Diacetate in Rats

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There is growing concern about the biological effects of organotin compounds widely distributed in the environment. Despite the large amount of toxicological data, only recently have the teratological and embryolethal effects of organotins on mammals been reported with the limited chemical species, for example, tri-*n*-butyltin compounds (Davis *et al.* 1987; Itami *et al.* 1990; Noda *et al.* 1991a), di-*n*-butyltin compounds (Noda *et al.* 1988; Ema *et al.* 1990), and triphenyltin compounds (Schatzow 1985; Noda *et al.* 1991b). In our previous studies, we have demonstrated that external malformations (cleft mandible, cleft lower lip, ankyloglossia and schistoglossia) and/or skeletal malformations (fused ribs, fused cervical vertebral arches and fused thoracic vertebral arches) occurred in rat fetuses after maternal exposure to di-*n*-butyltin diacetate (DBTA) throughout gestation (Noda *et al.* 1988) and during the period of organogenesis (Noda *et al. in press*). Similar observations with di-*n*-butyltin dichloride have been reported by Ema *et al.* (1991).

The present study was carried out to determine a critical gestational day of teratogenesis caused by DBTA in rats.

MATERIALS AND METHODS

DBTA was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Four-week-old Wistar rats of both sexes were obtained from CLEA Japan, Inc. (Tokyo, Japan), and were housed in a room maintained at a temperature of 23±2℃ and a relative humidity of 60±10% with a 12-hr light/dark cycle (light period: 7:00am-7:00pm). They were free access to feed (NMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Three-month-old female rats were paired overnight

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with single males of the same age, and the day on which sperm was observed in the vaginal smears was designated as day 0 of gestation.

In experiment 1, mated females were randomly assigned to 4 groups of 4-6 rats each. DBTA was dissolved in olive oil and given by stomach tube to pregnant rats at a dose level of 15 mg/kg in a volume of 2mL/kg for 2 or 3 consecutive days at 4 different periods of gestation (days 7-9, 10-12, 13-15, and 16-17). Every day, they were weighed and were observed for condition and behavior. Food consumption was also measured daily. At day 20 of gestation, all pregnant rats were sacrificed under ether anesthesia, and then the positions and numbers of living and dead fetuses, including resorbed fetuses, in the uterus were recorded. The number of corpora lutea and maternal thymus weight were also recorded. Uteri with total resorption were isolated and stained with 10% ammonium sulfide to determine the total number of implantations. Living fetuses were weighed and examined for their sex and the presence of external malformations. All of the living fetuses in each litter was fixed in 95% ethanol and examined for skeletal anomalies by staining the skeletons with the alizarin red S dye method (Dawson 1926).

In experiment 2, mated females were randomly assigned to 6 groups of 7-8 rats each. DBTA was given by stomach tube to pregnant rats at single doses of 15 and 30 mg/kg on 3 different days of gestation (day 7, 8, and 9). The other procedures were the same as experiment 1.

In experiment 3, mated females were randomly assigned to 6 groups of 9-10 rats each. DBTA were given by stomach tube to pregnant rats at doses of 5.0, 7.2, 10.5, 15.2 and 22.0 mg/kg on day 8 of gestation. Control rats received an equivalent volume of olive oil (2 mL/kg). The other procedures were the same as experiment 1.

Data were analyzed by Fisher's exact test. The litter was used as the statistical unit for calculation of fetal values.

RESULTS AND DISCUSSION

In experiment 1, treatment of pregnant rats with DBTA for 2 or 3 consecutive days at 4 different periods of gestation had no effect on body weights throughout the experiment, but reduced thymus weights on day 20 of

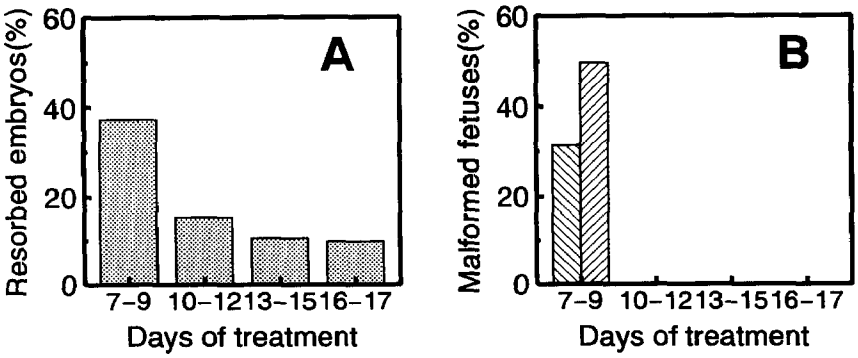

Figure 1. Incidence of dead or resorbed fetuses (A) and of fetuses with external (▨) and skeletal (▨) malformations (B) from the dams treated orally with DBTA (15 mg/kg) at different gestational stages.

gestation. In a group treated with DBTA on days 7-9, the number of living fetuses and their body weights were decreased (data not shown), and the incidence of dead or resorbed fetuses was greater than that in any other treated groups (Figure 1A). External malformations including cleft mandible, cleft lower lip, ankyloglossia, schistoglossia, exencephaly, facial cleft, etc. were observed in 4 of 5 litters with 31.5% of the fetuses (Figure 1B). These results were similar to those in our previous studies (Noda *et al.* 1988; Noda *et al. in press*). Skeletal malformations including anomalies of mandibular fixation, fused ribs, and fused cervical and thoracic vertebral arches, were observed in 4 of 5 litters with 49.7 % of the fetuses (Figure 1B). Cervical ribs, a minor skeletal abnormality, were observed in 69.9% of the fetuses. These observations were similar to those in our previous study in which pregnant rats were treated orally with DBTA during the period of organogenesis (Noda *et al. in press*). In contrast to this group, however, no fetus with external or skeletal malformation was observed in the other 3 groups treated with DBTA on days 10-12, 13-15 and 16-17.

From these results, it was found that the period when pregnant rats were most susceptible to DBTA-induced teratogenicity ranged from days 7 to 9 of gestation.

In experiment 2, when pregnant rats were treated with single doses of DBTA on day 7, 8 or 9 of gestation, the number of fetuses with external malformations was overwhelmingly increased by treatment on day 8 (Figure 2A). DBTA-induced malformations mainly consisted of cleft mandible, cleft lower lip, ankyloglossia and

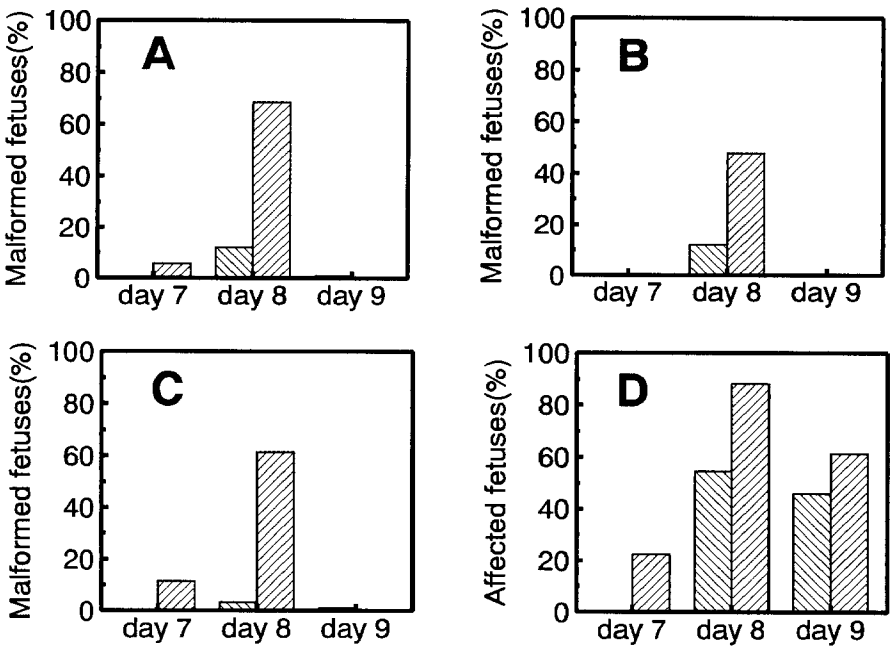

Figure 2. Incidence of fetuses with external malformations (A), with cleft mandible, cleft lower lip, ankyloglossia or schistoglossia (B), with skeletal malformations (C) and with cervical ribs (D) from the dams treated orally with DBTA at 15 (▨) or 30 (▨) mg/kg on 3 different days of gestation.

schistoglossia (Figure 2B). Only 3 of total 44 malformed fetuses were caused by treatment on day 7 or 9 of gestation. Two fetuses with peaked mandible or exencephaly and 1 fetus with agnathia were observed from the dams treated with DBTA on day 7 and 9 of gestation, respectively. The incidence of skeletal malformations was the highest in the fetuses from dams treated with DBTA on day 8 of gestation (Figure 2C). Twenty-eight of total 33 malformed fetuses were caused by treatment on day 8 of gestation. The types of skeletal malformations observed in this experiment were similar to those of *experiment 1*. Cervical ribs were observed in many fetuses from dams treated with DBTA on days 8 and 9 of gestation (Figure 2D).

From these results, the period when pregnant rats were most susceptible to DBTA-induced teratogenicity was limited to day 8 of gestation. However, the possibility that the critical gestational day of teratogenesis by DBTA may include not only day 8 of gestation but day 7 of gestation can not be ruled out, since DBTA administration to pregnant rats on day 7 of gestation

caused skeletal malformations with lower frequency.

In experiment 3, teratological effects of DBTA were examined when pregnant rats were given orally on day 8 of gestation at various dose levels. Maternal thymus weights on day 20 of gestation were unaffected with single doses of DBTA on day 8 of gestation. There were no significant effects of DBTA on the incidence of dead or resorbed fetuses, the number of living fetuses, and the body weights of living fetuses (data not shown). The incidence of malformed fetuses, however, was significantly increased at the highest dose of DBTA. As for external malformations, cleft mandible, cleft lower lip, ankyloglossia, schistoglossia, or exencephaly was observed in 2 and 18 fetuses from the dams treated with DBTA at 15.2 and 22.0 mg/kg, respectively. The fetuses with external malformations were found in 2 of 10 litters and 7 of 9 litters at 15.2 and 22.0 mg/kg, respectively (Table 1). In the skeletal observations, there was a significant increase in the incidence of malformed fetuses, such as anomalies of mandibular fixation, fused ribs, fused cervical and/or thoracic vertebral arches, and cranial hypoplasia, at the highest dose of DBTA. The fetuses with skeletal malformations were found in 5 of 9 litters at 22.0 mg/kg. The incidence of cervical ribs was increased at 15.2 and 22.0 mg/kg. Some studies suggested that an increase in skeletal variations, such as cervical ribs (Kato and Kitagawa 1974) and lumbar ribs (Yasuda and Maeda 1973), could be regarded as signals of teratogenic potential.

The external malformations observed in this study were mainly cleft mandible, cleft lower lip, ankyloglossia, schistoglossia, and exencephaly. According to Ferm and Carpenter (1968), severe facial malformations which involved cleft mandible were observed on the fetuses from dams injected intravenously with cadmium sulfate on day 7 of gestation in golden hamsters. However, such malformations as cleft mandible, cleft lower lip, ankyloglossia and schistoglossia are rare in the background data of teratology (Morita *et al.* 1987). Thus, the fetuses with these special malformations were observed from the dams treated with DBTA at more than 15.2 mg/kg on day 8 of gestation. It is well-known that the period of early differentiation which begins from about day 7 of gestation in the case of rats, is highly sensitive to teratogenesis (Wilson 1964). Rat embryo on day 8 of gestation begins to form mesoderm, neural plate and neural groove (Hiraiwa *et al.* 1960). Further studies, however, are necessary to clarify why mandibular anomalies are induced by treat-

Table 1. External and skeletal observations of fetuses from the dams treated orally with DBTA on day 8 of gestation.

	Olive oil 2 mL/kg	DBTA (mg/kg)				
		5.0	7.2	10.5	15.2	22.0
Fetuses/dams	115/9	140/10	138/10	120/10	117/10	103/9
External observations						
% of fetuses with malformations	0.9(1)	0	0.6(1)	0	1.9(2)	26.3(7)**
No. of fetuses with malformations	1(1)	0	1(1)	0	2(2)	18(7)**
Cleft mandible, cleft lower lip, ankyloglossia or schistoglossia	0	0	0	0	2(2)	14(7)**
Exencephaly	0	0	0	0	0	8(3)**
Cleft upper lip	0	0	0	0	0	4(1)
Peaked mandible	1(1)	0	0	0	0	0
Agnathia	0	0	0	0	0	1(1)
Microcephaly	0	0	0	0	0	1(1)
Vestigial tail	0	0	1(1)	0	0	0
Club foot	0	0	0	0	0	1(1)
Skeletal observations						
% of fetuses with malformations	0.8(1)	0	1.2(2)	0	0.7(1)	22.4(5)**
No. of fetuses with malformations	1(1)	0	2(2)	0	1(1)	13(5)**
Anomaly of mandibular fixation	0	0	0	0	0	9(5)**
Cranial hypoplasia	0	0	0	0	0	8(3)**
Fused ribs	0	0	0	0	0	6(1)*
Fused cervical or thoracic vertebral arches	0	0	0	0	0	5(1)*
Fused mandibula	1(1)	0	0	0	0	0
Agenesis of sacro-coccygeal or coccygeal vertebrae	0	0	2(2)	0	1(1)	0
No. of fetuses with cervical ribs	4(4)	3(2)	8(6)	9(4)	34(8)**	62(9)**

The litter was used as the statistical unit for calculation of fetal values. Thus, these values represent means of litter means within each group. ( ): No. of dams with the corresponding abnormal fetuses. *: Significantly different from control, $p<0.05$.
**: Significantly different from control, $p<0.01$.

ment with DBTA on day 8 of gestation.

In conclusion, a critical gestational day of teratogenesis by oral administration of DBTA to pregnant rats is considered to be day 8 of gestation without maternal toxicity. However, the possibility that day 7 of gestation is also involved in the critical day can not be ruled out, since DBTA exposure to pregnant rats on day 7 of gestation caused skeletal malformations in the fetuses with low frequency.

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